Volatiles modulate the development of plant pathogenic rust fungi

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Abstract Rust fungi are obligate biotrophic pathogens that differentiate a series of specialized cells to establish infection. One of these cells, the haustorium, which serves to absorb nutrients from living host cells, normally develops only in planta. Here, we show that the rust fungus *Uromyces fabae* (Pers.) Schroet. stimulates volatile emission of its host, broad bean (*Vicia faba* L.). Volatiles were identified and shown to be perceived by the fungus in in vitro assays that excluded the host. Three of them, nonanal, decanal, and hexenyl acetate promoted the development of haustoria on artificial membranes. In contrast, the terpenoid farnesyl acetate suppressed this differentiation. In assays using whole plants, farnesyl acetate reduced rust disease not only on broad bean but also on several cereals and legumes including soybean. This natural substance was effective against all rusts tested when directly applied to the host. This demonstrated that farnesyl acetate may serve as a powerful novel tool to combat rust fungi including

Phakopsora pachyrhizi that currently threatens the production of soybeans world-wide.

Keywords Fragrance · Haustorium · Morphogenesis · *Uromyces* · *Vicia* · Volatiles

Abbreviations

HMC Haustorial mother cell

GC/MS Gas chromatography/mass spectrometry

Introduction

Rust fungi are a species-rich group of highly aggressive plant parasites. Unlike many other pathogenic fungi, they do not kill their host, but they have evolved effective strategies to exploit living cells as feeding sources. The hallmark of this obligate biotrophic lifestyle is a highly specialized cell, the haustorium (from Latin haurire, “to suck”), first described by DeBary (DeBary 1863). It differentiates only in the interior of the leaf to complete a developmental program that starts with the germination of uredospores on the leaf surface and is followed by penetration through a stomatal opening (Fig. 1). Within the leaf, an infection hypha contacts a mesophyll cell where a haustorial mother cell (HMC) is formed, which begins host cell penetration and haustorium differentiation. The mature haustorium develops within the periplast of a living mesophyll cell and effectively absorbs nutrients without promoting host defenses (Staples 2000; Voegelé et al. 2001; Mendgen and Hahn 2002; Schulze-Lefert and Panstruga 2003). That this developmental program may depend on host-derived signals has been discussed for all infection structures including haustoria (Mendgen et al. 1996; Heath 1997). However, attempts to identify the

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Fig. 1 Outline of the development of *U. fabae* (dikaryon) on *V. faba*. After landing on a leaf, uredospores attach to the cuticle by formation of an adhesion pad. A germ tube grows toward a stoma guided by physical cues on the host surface. An appressorium forms that drives a penetration hypha into the substomatal cavity. Morphogenesis continues with the infection hypha that contacts a parenchymous cell. Shortly after, the hyphal tip differentiates the HMC, which in turn, produces the haustorium within the plant cell. At this point, the pathogen starts to absorb massive amounts of nutrients from the host, thereby establishing biotrophy

signals regulating the differentiation of the haustorium within the plant remained unsatisfactory.

Recent reports demonstrated that volatiles mediate the interactions of plants with insect herbivores (Picher-sky and Gershenzon 2002), with rhizobacteria (Ryu et al. 2003), and with fungi causing post-harvest fruit rots (Flaishman and Kolattukudy 1994; Kulakiotu et al. 2004). Furthermore, previous work indicates that host-derived volatiles are recognized as signals by plant-pathogenic fungi to regulate certain steps of development (Flaishman and Kolattukudy 1994; Staples and Hoch 1997). This motivated us to investigate whether volatiles may also influence the differentiation of rust haustoria. Here, we present key evidence that host volatiles indeed interfere with the regulation of this developmental step and farnesyl acetate can be used to control rust diseases in environmentally compatible production.

Materials and methods

Fungal and plant materials

Broad beans (*Vicia faba* L. cv ConAmore, obtained from Enza Zaden GmbH & Co. KG, Dannstadt, Germany) and the rust fungus (*Uromyces fabae* (Pers.) Schroet., isolate 12) were propagated and inoculated as described (Deising et al. 1991). Additional host plants used were *Phaseolus vulgaris* (cv Primel, obtained from Enza Zaden), *Medicago sativa* (cv Europe, obtained from KWS Saat AG, Einbeck, Germany), *Hordeum*

vulgare (cv Frankengold, obtained from Saatzucht Josef Breun GdbR, Herzogenaurach, Germany), *Triticum aestivum* (cv Kanzler, obtained from Saatzucht Engelen Büchling e.K., Germany), *Glycine max* (cv Erin, obtained from Pflanzenzucht Oberlimpurg, Schwäbisch Hall, Germany). Inocula included *U. appendiculatus* (isolate SWBR 1), *U. striatus* (isolate KN1-1), *Puccinia hordei* (isolate 22), *P. recondita* f. sp. *tritici* (race Arak), *P. graminis* f. sp. *tritici* (race ANZ), and *Phakopsora pachyrhizi* (race Thai 1). Plants were inoculated with a mixture of 50 mg of uredospores and 70 mg of milk powder in 100 ml of water. Even spore density on leaves and regular differentiation of appressoria over stomata were checked for each experiment by light microscopy.

Analyses of fungal differentiation in vitro and in planta

The infection process was studied in vitro on scratched polyethylene sheets (Rische and Herfurth GmbH, Hamburg, Germany; Deising et al. 1991) that were additionally misted with low gel-strength agar, 1% (Serva) and gelatin, 1% (Serva) in water. This resulted in droplets on the membrane that had a diameter of 200–400 μm . Spore density was adjusted in a way that hyphae had multiple contacts with each other by blowing 400 mg of uredospores into an inoculation tower covering membranes with a total surface of 40 $\times$ 60 cm. Pieces from these membranes (5 $\times$ 5 cm) were then immediately placed into air-tight sealed glass Petri dishes (100 ml volume) that provided high humidity through wet pieces of filter paper. Pure substances (Sigma-Aldrich) were diluted with diethyl ether (Merck) in steps of 1:10. Ten microliter of each dilution were dropped onto a filter paper dispenser (MN 615, Macherey-Nagel, Düren, Germany) which was attached to the upper lid of the Petri dish after evaporating the solvent (10 s). The volatile source was kept in place for 24 h. Each experiment was repeated at least three times.

The impact of volatiles on fungal growth in whole plants was studied with freshly inoculated, 10-days-old host plants kept in tightly closed 10 l glass cylinders for another 10 days (20°C, 16 h light, 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Every day, 0.5 μl of the pure substance was dropped with a syringe onto a filter paper through an opening in the glass cylinder. Since it was not possible to count the number of haustoria deep within the leaf or in the middle of a colony, 30 clearly visible HMCs at the edges of each of 10 colonies were examined by microscopy.

The effect of volatiles on rust disease was studied with 15-days-old whole soybean plants that had been inoculated 24 h ago with *P. pachyrhizi*. Plants were

sprayed with 1 ml of water or olive oil in which 1, 10 or 100 μl of farnesyl acetate were suspended. In an alternative treatment, farnesyl was mixed with 1 g of lanolin which was then distributed as a thin layer on the leaves by using a brush. Incubation was continued for 9 days in a growth chamber (air exchange every hour) that was adjusted to a light period of 16 h, at 22°C and 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a dark period of 8 h at 18°C.

Microscopy

Light microscopy was performed with a Zeiss Axioplan microscope using a 63 $\times$ NA 1.4 objective and a digital camera Zeiss Axiocam (Carl Zeiss AG, Jena, Germany). Fungal development on the leaf surface and within the leaf was studied after clearing the tissue with lactophenol (20%) over-night and staining with cotton blue (0.1%) in water for 2 h. For electron microscopy, the polyethylene membranes carrying the germlings were fixed in 2% glutaraldehyde followed by 1% osmic acid. Dehydration and embedding was via a graded ethanol/propylene oxide series and infiltration used Epon-Araldite resin via a modification of an established technique (Boehm et al. 1992). A drop of 100% resin was poured onto the polyethylene and covered with a sheet of mylar. A glass slide together with a lead weight put on it resulted in a flat, thin mount. The resin was polymerized for 18 h at 65°C. Next, the mylar and the slide were removed, the haustoria were selected by light microscopy and excised by punching a disk of 2 mm diameter out of the resin. Disks were mounted on blank resin blocks either with a cyanoacrylate glue or a two component glue. The block face was then trimmed to a trapezoid with the selected haustoria in the middle. Complete serial sections of haustoria were obtained using an Ultracut E microtome (Leica Mikrosysteme Vertrieb GmbH, Bensheim, Germany), and collected on copper slot grids with formvar films. Sections were stained with lead citrate/uranyl acetate and examined with a Hitachi H7000 electron microscope operated at 50 kV.

Identification of volatiles

Whole broad beans incubated at 20°C and 16 h day length under light of 270 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ were used for collection of volatiles. The aerial parts of the potted plants were enclosed in a flat-flange glass cylinder (50 cm $\times$ 12 cm $\varnothing$) fitting onto the top of the plastic pot that was tightly wrapped with aluminum foil to prevent contamination with soil-derived volatiles. The bottom of the cylinder was closed by two metal halves

which could be joined together and tightly fitted the stem of the plant just above the soil. The cylinder was covered with a single-necked flat-flange top attached to an air-circulation system containing charcoal traps (Donath and Boland 1995). Volatile collection was performed for 2 $\times$ 48 h followed by desorption of the carbon traps with 2 $\times$ 20 μl dichloromethane. The samples were analyzed by gas chromatography/mass spectrometry (Micromass MassSpec 2, Waters GmbH, Eschborn, Germany; Finnigan MAT Magnum, ThermoElectron GmbH, Bremen, Germany) and the eluting volatiles were identified by authentic references (Sigma-Aldrich).

Results

Effect of leaf volatiles on development of *U. fabae* in vitro

Previous work has shown that the germ tube of rust fungi recognizes the physical features of the outer ledge of the stomatal pore and starts penetration after differentiation of the appressorium (Hoch et al. 1987). In order to find the signal(s) responsible for haustorium differentiation, we spread out uredospores of the rust fungus *U. fabae* on polyethylene membranes imitating the hydrophobic host surface. At high humidity, uredospores germinated ($92 \pm 2\%$) and appressoria formation ($81 \pm 3\%$) was triggered by ridges that mimicked the stomatal ledges. However, this differentiation continued just to HMCs that were formed by $15 \pm 3\%$ of the uredospores inoculated. Additional coating of the surface of the membrane with miniscule agar droplets further improved differentiation of HMCs to $25 \pm 5\%$ but still without triggering the formation of haustoria. Other attempts to promote morphogenesis by modifying the membrane surface failed (data not shown). Therefore, we assumed that additional host-derived signals were essential for haustorial formation. To test whether or not volatiles might influence interactions between rust fungi and their hosts, we studied the development of germlings on polyethylene membranes in the presence of volatiles released from broad bean leaves. In the atmosphere of a closed test system (glass Petri dish) containing broad bean leaves without direct contact to the membrane surface, the differentiation of infection structures remained similar except for HMCs that differentiated haustoria ($0.8 \pm 0.3\%$) showing the same phenotype as those in planta (Fig. 2a). The effect became more pronounced ($6.0 \pm 0.5\%$) when the volatile-emitting leaves in the gas phase were previously inoculated with the fungus

(6 d.p.i.). Haustorial differentiation occurred only where HMCs had contacted neighboring hyphae (Fig. 2a). The differentiation of haustoria occurred only on membranes where high spore densities resulted in numerous contacts of HMCs with hyphae (results not shown). Haustorial development inside a hypha paralleled the natural infection process and the differentiation proceeded with formation of a haustorial neck with the typical neckband (Fig. 2b) and a structure resembling the extrahaustorial matrix sheathing the haustorial body (Fig. 2c) in mesophyll cells of the plant host. The enhanced differentiation in the presence of infested leaves suggested that infection might have, indeed, elicited the release of volatiles (Holopainen 2004), which the fungus then recognized as a cue for morphogenesis.

Identification of volatiles relevant for haustorium differentiation

Volatiles emitted from healthy and rust-infected plants were collected from intact plants in semi-closed sampling systems for four days after inoculation and analyzed by gas chromatography/mass spectrometry (GC/MS; Kunert et al. 2002). Infection did not generally alter the blend of fragrances released by broad bean but rather enhanced emission by a factor of about 10

Fig. 2 a–c Morphology of an in-vitro differentiated haustorium of *U. fabae* within a hypha from another uredospore stimulated by volatiles that were emitted from an infected leaf of *V. faba*. **a** Light micrograph of a haustorium. **b** Electron micrograph of the haustorial neck and **c** of the haustorial body. Bar 5 μ m (**a**), 1 μ m (**b**, **c**)

(Fig. 3a). We identified the volatiles and then used the pure synthetic substances to reproduce the observed effects in vitro and to assess their biological significance. Three of the major constituents of this volatile blend, decanal, nonanal, and hexenyl acetate, induced haustorial formation by HMCs on a membrane in a dose-optimum-correlation when introduced into the gas phase of a glass Petri dish (Fig. 3b–d). Combinations of these volatiles did not significantly increase the rate of haustorium differentiation (not shown). The fourth major volatile, farnesyl acetate, produced no apparent effect when individually employed (Fig. 3e). However, the compound counteracted haustorial development when applied simultaneously with decanal (Fig. 3f) or the other inducing compounds (not shown). Morphogenesis prior to haustorial development was not altered by these substances.

Effects of emitted volatiles on rust infections in planta

To examine the impact of these volatiles on fungal development in planta, we incubated intact broad bean plants immediately after inoculation with *U. fabae* in closed glass cylinders and provided the volatiles through evaporation from filter paper. In comparison to the control using only the solvent, exposure to decanal and nonanal significantly increased the number of haustoria that were produced by HMCs at the edge of a colony as observed by light microscopy of cleared leaf tissue samples (Fig. 3g). In contrast, farnesyl acetate application significantly decreased the number of haustoria. Microscopy gave no evidence for hypersensitive responses or other defense reactions like the appearance of necrotic cells that might have been caused by the treatments. Concurrent with the effects on haustorial numbers, the number of colonies rose by 76 and 132% for decanal and nonanal treatments, but declined by 48% for farnesyl acetate when compared to an untreated control (Fig. 4). This discovery was further evaluated in seven additional rust pathosystems, including three cereal rusts that cause severe epidemics. Not only the closely related species *Uromyces appendiculatus* and *U. striatus*, but also *P. pachyrhizi* infecting soybean, and even the cereal rusts *Puccinia graminis*, *P. hordei* and *P. recondita* responded to volatile treatment in the same way as *U. fabae* (Fig. 4). In addition to the number of colonies, also their size was affected by volatiles. This is demonstrated for *P. graminis* f. sp. *tritici* infecting wheat (Fig. 5a–c). Larger colonies resulted from nonanal, smaller colonies from farnesyl acetate (Fig. 5d), which is notable since colony size also affects the number of spores produced (Robert et al. 2004). Encouraged by these results we next

Fig. 3 **a–g** Volatiles from *V. faba* affect the differentiation of *U. fabae*. **a** Identification of volatiles by GC/MS from whole plants, infected by *U. fabae* and control. Compounds were identified by GC/MS using authentic references. Elution time is given in minutes. *IS* internal standard (1-bromodecane), *1* hexenyl acetate [(*Z*)-hex-3-enyl acetate], *2* decanal, *3* nonanal, *4* farnesyl acetate. **b–f** In-vitro differentiation of haustoria on polyethylene

membranes after exposure to authentic volatiles. Dilution factors are indicated on the *x*-axis. Data represent mean $\pm$ SD ($n = 600$ spores/variant). **g** In-planta differentiation of haustoria by HMCs at the edge of visible colonies after exposure to authentic volatiles. Values that differ significantly from controls are indicated by different letters on top of the columns (Dunnnett test, $P \leq 0.05$, $n = 300$ HMCs/variant)

investigated whether application of farnesyl acetate to infected soybean leaves in a well aerated growth chamber could inhibit the development of the pathogen in planta. Thus, soybean plants, inoculated with *P. pachyrhizi* 1 day earlier, were treated with different formulations of the compound. As carriers we used water, olive oil and lanolin, in which farnesyl acetate was sus-

pended or dissolved to reduce its evaporation. Spray application of farnesyl acetate mixed in water and or dissolved in olive oil reduced the colony numbers by up to 67 and 69%, respectively (Fig. 6). Application of farnesyl acetate as a thin lanolin coating which strongly retards evaporation of the compound caused a reduction of colonies of up to 98% (Fig. 6).

Fig. 4 Colony formation by other rust fungi in comparison with *U. fabae* after exposure to volatiles. For each pathosystem, colony counts from untreated controls were set to 100%. The farnesyl acetate treatment differed from the controls as indicated by the Dunnet test, $P \leq 0.05$. Data represent mean $\pm$ SD ($n = 400$ /variant)

Fig. 5 a–d Diameter of colonies from *Puccinia graminis* on wheat, exposed to volatiles for 6 days in closed glass cylinders. **a** Control. Treatment with farnesyl acetate (**b**) or decanal (**c**). **d** Colony diameter from the variants *a* to *c* (data represent mean $\pm$ SD, $n = 400$ /variant, ANOVA, $P \leq 0.05$)

Discussion

This study demonstrated the impact of induced, host-derived volatiles on rust fungus development on membranes and within the infected plant. Certain, fatty-acid-derived aldehydes, known to be involved in the plant's defense against pathogens (Feussner and Wasternack 2002), serve as positive signals for the

Fig. 6 a–c Colony formation by *P. pachyrhizi* on soybean treated with farnesyl acetate. Whole soybean plants were inoculated with *P. pachyrhizi*. 24 h later, the right halves of the leaves were coated with a thin layer of lanolin, the left halves remained untreated. **a** Lanolin only. **b** 10 μ l farnesyl acetate added per ml lanolin. Incubation continued for 9 days in a growth chamber. **c** Effect of carrier substance and farnesyl concentration on colony numbers. Data represent mean $\pm$ SD ($n = 12$ /variant). Values that differ significantly from the controls are indicated by different letters on top of the columns (Tukey–Kramer test, $P \leq 0.05$)

differentiation of haustoria of the fungus in vitro. We estimate that nanomolar concentrations of the volatiles are sufficient to induce this effect. A more precise assessment is not possible since it is unknown how much of the volatiles evaporated from the filter paper

during the set-up of the experiment. Importantly, we discovered that haustorial development is also negatively regulated by the volatile farnesyl acetate. This observation suggests that the plant derived volatiles are deeply involved in the signaling between *U. fabae* and broad bean and contribute to control the interactions between plants and rust fungi. A complex network of volatile signals seems to have evolved between rust fungi and their host plants, which, along with other (macro)molecular signals, govern the differentiation of specialized fungal cell types in order to establish the biotrophic interaction.

For several species of *Colletotrichum* it has been shown earlier that ethylene released from ripening fruits induced germination and appressorium formation on the host (Flaishman and Kolattukudy 1994). Plant volatiles do also play an important role during certain phases of the life of the more specialized obligate biotrophic rust fungi. Aldehydes and esters stimulate teliospore germination of *U. appendiculatus* (French et al. 1993) which ensures that germination preferably occurs in the vicinity of the host (Gold and Mendgen 1983). During the next phase of the life cycle, certain *Puccinia* species transform the leaf rosettes of their hosts into yellow “pseudo-flowers” that emit a strong scent (Raguso and Roy 1998). This in turn attracts insects to the infected parts of the plant where they coincidentally pick up pycnidiospores in order to support fertilization. In addition, the physical structure of the surface and fragrances emitted from the leaf seem to be important for the germination of uredospores and differentiation of germ tubes (French 1992; Epstein and Nicholson 1997; Staples and Hoch 1997). After penetration of the leaf surface, again both physical signals and volatiles appear to be important for the progression of morphogenesis. The differentiation of HMCs but not of haustoria of *P. graminis* can be induced in vitro by the volatile trans-2-hexen-1-ol (Wiethölter et al. 2003). We found for *U. fabae* that in vitro HMC differentiation could be accomplished by optimizing the physical features of the membrane surface. We used scratched polyethylene membranes that provided the hydrophobic surface for the germ tube and tiny ridges for the induction of appressorium formation. Additional minuscule agar droplets mimicked the conditions in the interior of the plant where cell surfaces are coated with the apoplastic fluid and thus are hydrophilic. This resulted in an improved differentiation of the infection hypha and the HMC. The addition of sugars and inactivated enzyme solutions that was reported to induce the development of haustorial initials in *Uromyces vignae* (Heath 1989, 1990) did not produce the anticipated effects in our system (data not

shown). However, the inclusion of volatiles into a closed incubation chamber resulted in haustorial development in vitro. Volatiles from *V. faba* leaves that were considerably induced after an infection by *U. fabae* provided positive signals for the differentiation of haustoria with regular haustorial bodies. Initial attempts, which used 22 other volatile substances which were previously reported to be induced by invading pathogens (Feussner and Wasternack 2002), remained unsuccessful (data not shown). Haustoria were only formed in neighboring hyphae. This result suggests that once the volatile signal is perceived, a quite unspecific surface is sufficient to initiate haustorial development. An appropriate uredospore density on the membranes facilitated regular contact of HMCs with neighboring hyphae and thus improved differentiation. Since farnesyl acetate acted as an antagonistic signal and strongly reduced the rate of differentiation, we first supposed that the volatile signal to regulate haustorium differentiation on membranes would be quite specific. However, experiments with several other rust fungi revealed that this volatile is also a potent inhibitor of colony development and possibly also of haustorium initiation for species infecting very different host plants. Therefore, farnesyl acetate may generally serve as a negative regulator during rust fungal development. Additional experiments are needed to explain whether this is the result of a direct effect of the volatile or the result of an inconspicuous plant defence reaction that is elicited by farnesyl acetate (Panstruga 2003). Investigations on the other obligate biotrophic plant pathogens, i.e., the powdery and the downy mildews, will reveal if these taxonomically unrelated groups may also respond to volatiles in the development of haustoria. We anticipate that these studies will provide insights into the convergent evolution of obligate biotrophy.

These findings allowed us to design novel approaches to control rust diseases in crops. Volatiles might either be externally applied as demonstrated here. In addition, the spraying of compounds that induce the release of anti-fungal volatiles from the plant may also prove useful in a protection scheme. Furthermore, plants may be genetically engineered to release altered volatile profiles. The feasibility of such an approach has been demonstrated (Lewinsohn et al. 2001; Vancanneyt et al. 2001; Kessler et al. 2004). Transgenic plants producing increased levels of farnesyl acetate or derivatives thereof could provide more durable field resistance against rusts than is currently available. Such a defense is highly needed especially against the soybean rust *P. pachyrhizi*, which has recently spread beyond its original range in eastern

Asia and Australia into Africa, Central America, and South America where it had caused losses of \$2 billion just in Brazil in 2003 (Pivonia and Yang 2004; Stokstad 2004). This pathogen, which also represents a major threat to soybean production in the USA has arrived in five southern states (Miles et al. 2003; Rogers and Redding 2004; Stokstad 2004). Given that several rust species attack other important crops like wheat and barley, cultivars that carry a trait for general resistance against rusts would be extremely desirable. Our data suggest that host volatiles perceived by rust fungi may represent such traits.

In summary, we identified host-derived volatiles as novel signals that are recognized by rust fungi to regulate haustorial development. Nonanal, decanal, and hexenyl acetate stimulated this essential part of pathogenesis whereas farnesyl acetate blocked it. External application of farnesyl acetate inhibited disease in all rust pathosystems tested. In the future, volatiles may serve as a powerful novel tool to combat rusts.

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